Organization and Expression of Plasmodial Genes Required for Erythrocyte Invasion

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Survival of the malaria parasite within its vertebrate is the result of multiple highly evolved mechanisms which allow the parasite to modulate or evade host defenses. Principle among these mechanisms is the ability to minimize contact between the parasite and the host immune system by remaining intracellular for the majority of its asexual life-cycle. For P. falciparum, invasion of host erythrocytes, as well as the sequestration of infected erythrocytes in the microvasculature, represent specialized mechanisms which are dependent upon receptor-ligand interactions between the parasite and host cells. As such these interactions represent models by which to address questions of cell-cell interactions at the molecular level and which represent rational sites for therapeutic intervention. The research focus of the laboratory was aimed at defining the biochemical and genetic basis for merozoite invasion of erythrocytes and sequestration of the infected erythrocyte. Studies focussed on the organization

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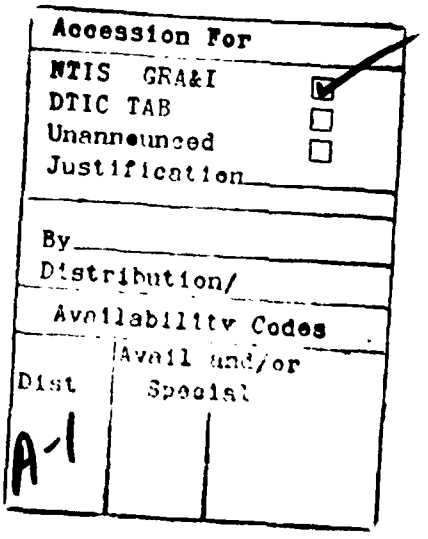
and expression of 2 P. falciparum genes involved in these mechanisms: the glycophorin binding protein (GBP 130) and the knob-associated histidine-rich protein (KAHRP) genes. Further insight into the function of the KAHRP gene was obtained through the analysis of spontaneous, non-revertable mutants which have lost the expression of this gene (K^- parasites). Molecular analysis of these mutants revealed a novel mechanism for the generation of chromosomal polymorphisms in this organism. These observations have been generalized through the discovery and characterization of similar events on chromosomes 1 and 8. The contribution of these rearrangements to the regulation of gene expression was investigated in transcription studies of these genes. These studies provide insight into the physiologically important pathway of antigenic variation in this organism.



SUMMARY

The goals of this proposal were to define the structure and function of genes implicated in P. falciparum invasion of erythrocytes and the sequestration of those infected erythrocytes in the microvasculature. The gene for the glycophorin binding protein of 130,000 dalton was cloned[b], the complete nucleotide sequence of the cDNA and gene defined[c] and the expression of that gene in E. coli was achieved, generating the substrates for biochemical studies. Previous studies by Perkins defined this protein on the merozoite surface as involved in erythrocyte invasion. Antibodies against the native protein inhibited the re-invasion of erythrocytes by merozoites in vitro. The primary sequence of this protein was predicted to encode 11 copies of a 50 amino acid repeat sequence, preceded by a 225 amino acid amino terminal charged domain. While the role of tandem repeat sequences in plasmodial genes is still enigmatic, a correlation between structure and function for this gene was possible. Through the overexpression and purification of recombinant protein expressing between 3-11 repeats, it was found that the strength of binding of the protein to its ligand, glycophorin, was a function of repeat number[c]. Thus, maximal binding to glycophorin was achieved only when 11 copies of this repeat was expressed. The repeats are well conserved within the molecule as well as for different strains of P. falciparum, suggesting that a critical function for the repeat sequence has been selected. Antibodies raised against the recombinant protein (monoclonal and polyclonal) inhibited the invasion of erythrocytes by merozoites in vitro[c]. Studies were pursued in collaboration with Col. Jeffrey Chulay (WRAIR) to assess the significance of this molecule in mediating invasion in vivo, utilizing the Aotus monkey model.

Studies on sequestration focussed on the isolation and characterization of the gene for the knob associated histidine rich protein. Through the use of a genomic clone isolated and characterized for an evolutionarily conserved protein in an avian parasite, the histidine-rich protein of P. lophurae[a], cDNA clones were isolated from a P. falciparum library constructed to mRNA isolated from the knob-expressing isolate FCR3[e]. The complete sequence of the predicted coding region of this protein as well as 3' and 5' non-coding sequences was determined and revealed a 634 amino acid sequence rich in lysine and histidine and containing three distinct, tandemly repeated domains. Indirect immunofluorescence using affinity purified monospecific antibodies directed against recombinant protein expressed in E. coli localized the KAHRP to the membrane of knobby infected erythrocytes. Immunoelectronmicroscopy established that the protein is clustered on the cytoplasmic side of the erythrocyte membrane and is associated with the electron dense knobs. Experiments to define

the role of this protein in mediating cytoadherence were attempted by investigating the interactions between this protein and other knob associated proteins.

The role of the KAHRP in formation of the knob structure was investigated through the analysis of mutations which lead to the loss of knob formation. K^- mutants arise spontaneously in culture, and can be cloned out of natural infections. The molecular basis for this mutation was investigated in four clonal, K^- isolates. In all cases analyzed, the gene for the KAHRP had undergone a rearrangement, resulting in the deletion of 3' coding sequences[d]. The chromosome to which this gene maps, chromosome 2, had undergone a rearrangement in these mutants, resulting in a telomeric location for the truncated KAHRP.

A general method was developed for determining the structure of the rearranged chromosomes using the polymerase chain reaction (PCR) and direct sequencing, in order to avoid further gene rearrangements in bacteria. Using this approach the breakpoints for these K^- isolates was determined and found to be the result of a chromosome breakage and healing event, leading to a truncated chromosome[f]. The generality of this unusual pathway for generating null mutations and chromosome polymorphisms was investigated by developing a strategy for identifying other polymorphic breakpoints. This strategy resulted in the identification of two additional genetic loci which had undergone chromosome breakage and healing to generate null alleles and polymorphic chromosomes. Using the strategy developed for the KAHRP, K^- mutants, the sequences of these breakpoints were determined[f,g]. A common sequence element was found to be conserved at these breakpoints, indicating a specificity to this process of breakage and healing[g]. The effect of this mechanism on the expression of the KAHRP gene was analyzed by developing transcriptional assays for plasmodial genes[h]. The isolation and stabilization of upstream promoter sequences in prokaryotic vectors has proven to be a daunting task; nonetheless the complete structure of one such element was determined[i]. These studies provide the basis for the characterization of the mechanism of the regulated expression of plasmodial genes.

PUBLICATIONS

a. Ravetch, J.V., Feder, R., Pavlovec, A. and Blobel, G. (1984) Primary structure and genomic organization of the histidine-rich protein of the malaria parasite P. lophurae. Nature 312: 616.

b. Ravetch, J.V., Kochan, J. and Perkins, M. (1985) Isolation of the gene for a glycophorin-binding protein implicated in erythrocyte invasion by a malaria parasite. Science 227: 1593.

c. Kochan, J., Perkins, M. and Ravetch, J.V. (1986) A tandemly repeated sequence determines the binding domain for an erythrocyte receptor binding protein of P. falciparum. Cell 44: 689.

d. Pologe, L.G. and Ravetch, J.V. (1986) A chromosomal rearrangement in a P. falciparum histidine-rich protein gene is associated with the knobless phenotype. Nature 322: 474.

e. Pologe, L.G., Pavlovec, A., Shio, H. and Ravetch, J.V. (1987) Primary structure and subcellular localization of the knob-associated histidine rich protein of P. falciparum. Proc. Natl. Acad. Sci.

f. Pologe, L.G. and Ravetch, J.V. (1988) Large deletions result from breakage and healing of P. falciparum chromosomes. Cell 55: 869.

g. Pologe, L.G., de Bruin, D. and Ravetch, J.V. in preparation.

h. Lanzer, M. and Ravetch, J.V. in preparation.

i. Lanzer, M. and Ravetch, J.V. in preparation.

REVIEWS

j. Ravetch, J.V., Young, J. and Poste, G. (1985) Molecular genetic strategies for the development of anti-malarial vaccines. Biotechnology 3, 729.

k. Perkins, M. and Pavetch, J.V. (1985) Interaction of P. falciparum merozoite proteins with the erythrocyte surface. In Vaccines, Cold Spring Harbor Press, N.Y.

l. Ravetch, J.V. (1989) Chromosomal polymorphisms and gene expression in Plasmodium falciparum. Experimental Parasitology 68: 121.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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INTRODUCTION

The malaria parasite, Plasmodium falciparum, has evolved numerous strategies to evade host immune defenses. Among these strategies, the invasion of host erythrocytes, the sequestration of these infected cells from the filtering action of the spleen and the antigenic variation manifestated by different isolates are key components to parasite survival. Each of these mechanisms offer attractive targets for therapeutic intervention in the life-cycle and virulence of this pathogen.

Invasion of erythrocytes by P. falciparum is a receptor-mediated event, dependant upon specific sialic-acid rich glycoproteins, the glycophorins, on the surface of erythrocytes. Parasite invasion of erythrocytes deficient in these proteins is greatly reduced (1). Antibodies to specific domains of glycophorin A as well as tryptic fragments of glycophorin A block invasion (2-4). Since other species of malarial parasites do not invade human erythrocytes or do not interact with glycophorin, it is likely that there are specific parasite proteins that recognize human glycophorin. When these studies were initiated in 1985, a glycophorin binding protein of 130,000 daltons had been identified (5) and found to be localized to the surface of merozoites (6). Antibodies against this parasite protein were further shown to block merozoite invasion of erythrocytes, in vitro (6). The role of this protein was critically assessed through the molecular cloning of the gene for this protein. Purified recombinant protein was obtained from the overexpression of this gene in E. coli and used to study its binding to glycophorin. Finally, the recombinant protein was evaluated for its ability to elicit protective immunity in a monkey immunization trial. More recent evidence (7) has suggested that the terminal sialic acid residues of glycophorin are critical for parasite binding to human erythrocytes. Parasite proteins which bind to this domain include the major merozoite surface antigen gp195 (8), as well as other erythrocyte binding proteins (9).

The significant mortality associated with falciparum malaria results, in part, from the blockage of cerebral vessels by infected erythrocytes which leads to focal anoxia in brain tissue. This selective accumulation of infected erythrocytes appears to be an adaptive mechanism for the parasite by allowing the infected cell to avoid splenic clearance and favoring parasite development in a low pO_2 environment (10). Cytoadherence between the infected erythrocyte and the endothelial cell is dependant upon specific parasite and host molecules, including a specialized structure on the surface of the infected erythrocyte, the knob. Ultrastructural studies on both in vivo and in vitro cytoadherence models suggested that knobs were a focal point for the adhesive interaction (11,12). Spontaneous mutants of P. falciparum which lacked knobs did not

cytoadhere in vitro (13) or sequester in vivo (14), suggesting that the knob structure played a role in the cytoadherence interaction. Other parasite and host proteins had been defined which were necessary to the cytoadherence interaction, including thrombospondin (15), the determinant recognized by the OKM5 antibody, now classified as CD36 (16) and high-molecular weight, strain-specific parasite proteins, referred to as the Pf EMP-1 family (17). Studies to investigate the role of the knob structure in parasite physiology focussed on defining the structure of a parasite protein which is a necessary component of this structure, the knob-associated histidine rich protein (KAHRP). The gene for this protein was cloned, its primary structure determined and overexpressed in E. coli to generate pure recombinant protein. Monospecific antisera were raised against this protein and localized the KAHRP to the cytoplasmic face of the knob structure, confirming its physical association with that parasite complex. A series of studies were undertaken to determine whether the KAHRP can interact with thrombospondin, another molecule implicated in cytoadherence.

The loss of knobs resulted in parasites which failed to cytoadhere. These mutants were characterized to determine the mechanism by which the knob structure was lost. The KAHRP was found to be absent in these mutants, with no detectable mRNA present for this gene. The molecular basis for this null phenotype was shown to result from a DNA rearrangement in the KAHRP gene, involving a terminal deletion of the gene and the distal portion of chromosome 2. The net result of this rearrangement was a truncated chromosome, explaining the polymorphisms observed for this chromosome in different geographic isolates of the parasite. Strain-dependant antigenic variation had been noted for P. falciparum and is correlated with the pathogenicity of the parasite in endemic areas. The observation that chromosomal polymorphisms are strain-dependant and occur with high frequency (18) suggested that this genetic pattern is involved in the mechanism of antigenic variation and may play a role in the adaptation of the parasite to immune pressure. With the discovery of a chromosome 2 polymorphism in the KAHRP gene, an opportunity was presented to define this class of chromosomal polymorphisms at the molecular level through the characterization of the parental and rearranged chromosomes. A general method was developed to define these chromosomal breakpoints, avoiding the propagation of these sequences through secondary hosts. The structure of these breakpoints revealed a non-random process of breakage and healing through the direct addition of telomeric repeats.

To determine the generality of the novel mechanism of breakage and healing to the generation of chromosomal polymorphisms in P. falciparum, a strategy was developed to identify other genes whose rearrangements resulted in null phenotypes with accompanying chromosomal polymorphisms. Two

additional examples were identified on chromosomes 1 and 8, confirming the general nature of this mechanism. These rearrangements were isolated and structurally defined. A consensus sequence was found at the breakpoint, indicating a specificity to either breakage or healing. Examples of breakage and healing in protozoans have been described for ciliated Tetrahymena and Paramecia. In those organisms, a developmentally regulated process of chromosomal fragmentation, followed by the addition of telomeric repeats results in the formation of the transcriptionally active macronucleus, from a silent micronucleus (19). The kinetoplastids have also provided evidence that gene rearrangement to telomeric sites can result in transcriptional regulation of the translocated gene (20). Studies were therefore initiated to determine the effect of this mechanism of chromosome breakage and healing on gene transcription in P. falciparum.

No studies had been published on gene transcription in P. falciparum, despite the importance of transcriptional regulation to gene expression in this organism. This is due to the difficulty in 1) obtaining transcriptionally active nuclei to assess RNA polymerase density and hence, transcriptional activity and 2) the instability of falciparum DNA in prokaryotic hosts in regions residing outside of coding domains, probably the result of the >90% A+T content of these sequences. Beginning in 1988, the laboratory undertook to attack both these problems in order to test the effect of chromosome rearrangement on gene expression and to define the cis and trans acting elements necessary for gene expression in P. falciparum. Such studies are a necessary pre-requisite to developing a transformation system for this organism, a crucial methodology in the analysis of the physiology of this parasite.

Personnel involved during this period were:

Jeffrey V. Ravetch,MD,PhD.	Associate Member/P.I.
Jarema Kochan, Ph.D.	Postdoctoral Fellow
Michael Lanzer, Ph.D.	Postdoctoral Fellow
Laura G. Pologe, Ph.D.	Postdoctoral Fellow
Mary-Ann Zalman, Ph.D.	Postdoctoral Fellow
Martha Till, M.S.	Graduate Student
Derik de Bruin, B.S.	Graduate Student
Brian Rubin, B.S.	M.D.-Ph.D. student
Mark Samuels, M.S.	Research Assistant
Helen Valsamis, B.S.	Research Assistant
Marta Bolstad, B.S.	Research Assistant

PROGRESS REPORT

Studies on the Glycophorin Binding Protein (GBP-130) 1985-1986

To assess the role of this parasite surface protein in erythrocyte invasion the gene encoding this protein was isolated and characterized. A rabbit antisera, generated to the GBP130 defined by Perkins (6), was used to screen a cDNA library constructed to FCR-3 asynchronously growing parasites in the expression vector pUC-9. Positive clones were confirmed by Western blot analysis of the E. coli expressed fusion proteins. The primary sequence of the partial cDNA clones revealed a tandemly-repeated sequence of 50 amino acids. Antibodies raised to the E. coli expressed fusion protein detected the GBP-130 as determined by Western blot of parasite extracts and indirect immunofluorescence on P. falciparum infected erythrocytes (Ravetch, Kochan and Perkins, Science 227, 1593 (1985)).

Complete characterization of this gene was undertaken through the isolation of overlapping cDNA clones and the corresponding genomic clone. 2924 nucleotides of sequence was determined, which encoded the full length protein and portions of the 5' and 3' untranslated sequences. A hydrophobic NH_2 signal sequence was found, followed by a 225 amino acid domain consisting of 35% basic residues. The remainder of the predicted protein sequence consisted of 11 copies of a 50 amino acid repeat. Analysis of multiple geographic isolates of P. falciparum revealed that the GBP-130 was highly conserved at the protein and nucleic acid level. To study the role of the unusual repeated domain in this protein, recombinant proteins expressed in E. coli containing 3.5, 4.5, 5.5 and all 11 repeats were generated. The binding of these proteins to a glycophorin affinity matrix was studied. The result of those studies indicated that the 50 amino acid repeat is a binding site for the glycophorin affinity matrix and that the degree of binding is increased with increasing numbers of repeats. Antibodies generated to these repeats were shown to inhibit merozoite invasion of erythrocytes by 73% (Kochan, Perkins and Ravetch, Cell 44, 689 (1986)).

Based on these studies, an in vivo evaluation of the GBP-130 in merozoite invasion was undertaken in collaboration with Col. Jeffrey Chulay of WRAIR. Recombinant GBP-130, expressing 3.5 repeats, was purified at Smith, Kline by Jim Young and used to immunize 3 Aotus monkeys. Serum titers revealed high levels of antibodies to the recombinant protein. Immunized and control animals were then challenged with parasites and evaluated for a protective response. No protection was observed in this study (Chulay, personal communication). Further evaluation of the GBP-130 protein in vivo has not been pursued.

Studies on the Knob Associated Histidine Rich Protein (KAHRP)

Primary structural analysis of the gene and protein 1986-1987

A histidine rich protein had been described by Kilejian (21) which was associated with the knobby phenotype. To evaluate the role of this protein in the knob structure and its function in cytoadherence, cDNA clones were isolated for this molecule. These clones were identified in a FCR-3 cDNA library constructed to trophozoite RNA by homology with a histidine rich protein gene isolated from the avian parasite P. lophurae (Ravetch, et al. Nature 312, 616 (1984)). Identical cDNA clones were identified using a cDNA probe constructed by subtractive hybridization between K^+ and K^- isolates. These cDNA clones detected a 4.2 kb mRNA in K^+ but not K^- isolates. Antibodies raised against the recombinant protein expressed in E. coli recognized a protein of 80,000-90,000 relative molecular mass in knobby but not knobless isolates. The protein and gene were conserved in multiple geographic isolates. The primary sequence encoded a 634 amino acid protein, rich in lysine and histidine and containing three distinct tandemly repeated domains. Indirect immunofluorescence, using affinity purified monospecific antibodies directed against the recombinant protein synthesized in E. coli, localized the KAHRP to the membrane of the knobby-infected erythrocytes. Immunoelectron microscopy established that the protein is clustered on the cytoplasmic side of the erythrocyte membrane and is associated with the electron-dense knobs (Pologe, Pavlovec, Shio and Ravetch, PNAS 84, 7139 (1987)).

Studies on the interaction of KAHRP with thrombospondin 1986-1988

The significance of the knob is its ability to mediate cytoadherence. The KAHRP may function in the assembly of the knob or in cytoadherence. Studies by Roberts (15) suggested that thrombospondin (TSP) played a crucial role in cytoaderence through its interaction with the infected erythrocyte membrane. TSP was shown to bind to both a histidine-rich glycoprotein in human sera and to endothelial cell molecule defined by OKM5 (22,23). A model for the possible role of the KAHRP in cytoaderence was suggested by its homology to the histidine-rich glycoprotein of human serum. Studies were undertaken to test the ability of the recombinant KAHRP to bind to TSP, in collaboration with Ralph Nachman (CUMC). Two types of binding studies were undertaken: 1) An affinity matrix was prepared with the recombinant KAHRP and radiolabelled TSP used as a ligand; 2) KAHRP was immoblized on plastic surfaces and unlabelled TSP bound to the coated surfaces was detected by an ELISA using anti-TSP. Purification of soluble KAHRP proved to be exceedingly difficult, resulting in insoluble protein from E. coli.

Preliminary experiments suggested that the recombinant KAHRP could bind to TSP, but the difficulties in obtaining soluble protein made conclusive statements impossible. Despite considerable efforts, a general method to obtain highly purified, soluble KAHRP in the E. coli system was not achieved. Further studies along these lines were not pursued when experiments performed on CD36 demonstrated that molecule mediated the binding of infected erythrocytes in the absence of TSP and could not be blocked by antibodies to TSP (24,25).

Chromosomal rearrangements in the KAHRP gene in knobless isolates 1986-1987

Spontaneous loss of the knobby phenotype occurs both in vivo and in vitro, resulting in parasites which cannot cytoadhere and are consequently unable to avoid splenic clearance. The molecular basis of this non-revertable mutation was investigated in three knobless clones of FCR-3. In all cases, the KAHRP mRNA was not detectable, accounting for the loss of protein accumulation. The genetic basis for this failure to accumulate mRNA for this gene was studied by characterizing the KAHRP gene and chromosome on which it resides. Surprisingly, the gene had undergone a rearrangement in all cases studied, resulting in deletion of distal coding sequences. A chromosomal polymorphism was associated with this knobless phenotype, consistent with a 50 kb deletion of chromosome 2. Direct evidence that the chromosomal polymorphism was linked to the KAHRP rearrangement was demonstrated by Bal 31 nuclease sensitivity of the knobless versions of chromosome 2. Those results indicated that a terminal deletion had occurred in chromosome 2, relocating the KAHRP gene to a chromosome end. This was the first molecular characterization of a chromosomal polymorphism in P. falciparum (Pologe and Ravetch, Nature 322, 474 (1986)).

These studies raised several important questions:

1) What is the mechanism of this terminal deletion?

Two models were possible based on these data - telomere exchange, resulting from reciprocal recombination between homologous sequences in the KAHRP gene and a chromosome end or chromosome breakage with direct addition of telomeric sequences to result in a healed chromosome.

2) How general is this mechanism terminal deletion in generating chromosomal polymorphisms in P. falciparum?

Concurrent studies had demonstrated that the 14 chromosomes of P. falciparum were highly polymorphic, varying in a strain-dependant manner. Those studies supported the view that the genetic basis for strain variation was linked to the chromosomal polymorphisms observed. Characterization of the mechanism of these polymorphisms was consequently of importance.

3) What is the consequence of rearrangement on gene transcription?

The loss of accumulation of mRNA for the KAHRP in knobless

mutants could be the result of transcriptional repression of the gene in its novel chromosomal position or the instability of a truncated mRNA. Distinguishing between these possibilites would be important in determining the role of these rearrangements in parasite physiology.

4) What is the selection to maintain a mechanism which can result in a non-viable phenotype and irrevocable gene loss in a haploid organism?

This question is perhaps the most intriguing, since the selective pressure would be to maintain knobs and cytoadherence. The consequence of terminal deletion is the loss of 50 kb from chromosome 2, during the haploid stage of the parasite. Thus, it would appear that maintainance of this mechanism of terminal deletion would argue for other selective forces favoring these genetic rearrangements.

Structure of the KAHRP gene in K^+ and K^- parasites 1986-1988

To distinguish between the models presented in question (1), the breakpoints of the rearrangements in the KAHRP gene in the knobless isolates FVO^-,D3,D4 and E96 had to be defined and compared to the wild-type sequence of the gene. Cloning of these breakpoints initially relied upon the ability of Bal 31 to render the rearranged chromosome 2 accessible to cloning in an E. coli host. That approach was used and some sequence information was obtained. However, it soon became apparent that the highly repetitive sequences found at chromosome ends were highly recombinogenic in E. coli and were subject to deletions and rearrangements upon cloning. Another method had to be developed which would allow analysis of the parental and rearranged chromosomes avoiding the ambiguity introduced by propagation in bacteria. A general method was developed which avoided cloning in bacteria, through the use of the polymerase chain reaction (PCR). DNA fragments were directly amplified from the genomic DNA of knobby and knobbless isolates using primers which flanked the breakpoint, as detemined by restriction mapping studies. An oligonucleotide complementary to the telomeric repeat of P. falciparum was used to specifically target the breakpoints. This sequence was detemined from the information obtained from the Bal 31 clones. Direct DNA sequencing was then performed on the amplified fragments, using both enzymatic and chemical degredation methods.

Using this powerful technique, the breakpoints of four knobless isolates was determined. Three had the identical sequence (D3, D4 and E96) while the fourth was different (FVO^-). However, in all cases, an abrupt transition was seen from KAHRP sequences to the simple telomeric repeat GGGTTTA. No subtelomeric sequences were found, nor any other target for homologous recombination. The sequences distal to the breakpoint, determined out to 15 kb, were absent from the genome of the knobless isolates. These studies strongly suggested that

telomeric conversion mediated by reciprocal recombination was not the mechanism generating these rearrangements. Rather, chromosome breakage and healing was the model more compatible with the data. The sequences at the breakpoint appeared to be non-random, with conserved sequence elements found. In particular a CA dinucleotide preceded the telomere addition site in all cases, suggesting that it may be part of a larger consensus sequence mediating specific breakage and healing (Pologe and Ravetch, Cell 55, 869 (1988)).

Generality of breakage and healing in generating chromosomal polymorphisms in P. falciparum 1987-1988

The terminal deletion in chromosome 2 resulted in the loss of expression of the KAHRP. This observation provided the strategy for screening for other chromosome polymorphisms. The assumption was made that like the KAHRP, other defined antigens might be the target site for breakage and healing, with concommitant chromosomal polymorphisms. A screen was established to identify strains in which the loss of a specific antigen was associated with a chromosomal polymorphism. Screening was done at both the protein and RNA levels. Probes for 10 cloned antigens were used to screen the RNA isolated from 18 different strains of P. falciparum from different geographic origins. Two additional examples were discovered. The HRP II mRNA was not expressed in strain D10 and was associated with a polymorphism on chromosome 8. Similarly, the RESA gene was not expressed in the FCR-3 derived clones A2, D3, D4, E96 and clone 1 with a polymorphism identified on chromome 1. These two examples were characterized to determine if the loss of expression was the result of a DNA rearrangement in the corresponding gene, relocating the gene to a chromosome end with a accompanying terminal deletion.

Restriction mapping studies were performed on HRP II in strain D10 and RESA in strain A2, as described for the KAHRP gene in strain D3. In both HRP II and RESA, the genes were found to be rearranged in the non-expressing strains, resulting in truncated DNA fragments appearing heterogeneous, indicative of the presence of telomere repeats. Confirmation of this structure came from Bal 31 studies on these genes in both expressing and non-expressing strains. Once again, the increased sensitivity to this precessive exonuclease confirmed that these genes were telomerically associated in the non-expressing strains. Precise characterization of the HRP II gene in expressing and non-expressing strains was accomplished by the combination of cloning of the Bal 31 generated fragments and PCR amplication of the breakpoint (Pologe and Ravetch, Cell 55, 869 (1988)).

The RESA gene could not be amplified in this direct strategy, suggesting that a more complex rearrangement might have occurred at the breakpoint (Pologe, de Bruin and Ravetch, in preparation). Subsequent studies (Pologe, de Bruin and Ravetch,

submitted (1989)) confirmed that the RESA gene rearrangement involved a DNA inversion which preceded the breakage and healing event.

The structure of these rearrangements confirmed the model suggested by the KAHRP studies, that breakage and healing accounts for the chromosomal polymorphisms observed for these chromosomes and results in the loss of gene expression of the gene at the site of rearrangement. The consensus sequence found at the breakpoint was confirmed by these studies, further indicating that specificity was involved in breakage and/or healing.

Effect of breakage and healing on transcription 1987-1988

In all cases examined, the breakage and healing events which result in chromosome polymorphisms and null phenotypes showed no evidence of RNA accumulation. In the case of the HRP II and RESA genes, the orientation of the genes relative to the centromere of chromosomes 8 and 1, respectively, resulted in the loss of the upstream sequences in the rearranged allele with the presumptive promoter sequences. The loss of RNA accumulation could be explained in those cases by the deletion of the promoter elements. However, a promoter had yet to be defined for P. falciparum and no information was available on transcriptional initiation sites, on termination, processing or RNA stability in this organism. Such basic studies had to be performed to begin to address the effect of rearrangement on such processes. For the KAHRP gene, upstream sequences were retained in the rearranged alleles. Was the gene transcribed?

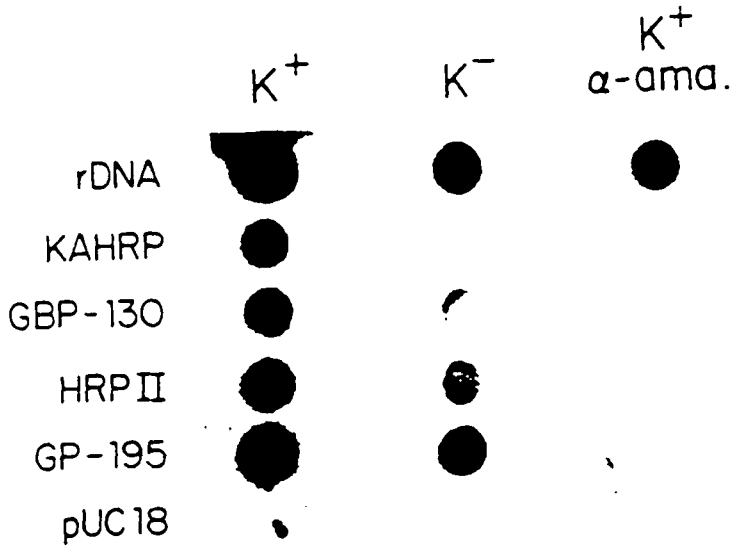
Studies were initiated during this period to address these basic questions of parasite biology. Transcription studies were performed by developing a general method for isolation transcriptionally active nuclei from infected erythrocytes. These nuclei could then be allowed to incorporate radiolabelled UTP into elongating RNA chains, as a measure of RNA polymerase density. Such studies were performed on knobby and knobless isolates, comparing transcription of the KAHRP gene to the GBP130 gene, the gp195 gene, the HRP II gene and the ribosomal genes. The transcription of these genes was tested for sensitivity to the RNA polymerase II inhibitor α-amanitin. All of these genes were transcriptionally active in knobby isolates. α-amanitin arrested transcription of the KAHRP, the GBP130, the gp195 and the HRP II but not the ribosomal genes, indicating that the ribosomal genes are transcribed by a different polymerase (pol I) and the antigen genes are transcribed by a pol II RNA polymerase of the eukaryotic type. Despite the fact that the upstream sequences for the KAHRP are intact in knobless isolates, the gene is not transcribed, indicating that the proximity of the telomeric repeats influences the ability of RNA polymerase II to transcribe this gene. These results are presented in Figure 1 of

the appendix (Lanzer and Ravetch, in preparation).

These results raised the question of what is the structure of a P. falciparum promoter. Is transcription continuous or discontinuous? Are consensus cis acting elements present in the upstream regions of plasmodial genes? Since the definition of a promoter is a functional one, the ultimate demonstration of such elements will depend on an in vivo or in vitro transcription assay. Studies were initiated in 1987-1988 to begin to address these questions. The structure of the transcription unit for the gp195 was first determined. The nucleotide sequences extending 1500 nucleotides upstream of the translational start site were cloned and their primary structure determined. It is important to note that the extraordinary A+T content of these sequences make them particulary unstable in E. coli, and are subject to rapid deletions and rearrangement. Complete characterization of these sequences relied upon the isolation of multiple, partially deleted clones and PCR techniques to fill in gaps in the clones. The composite sequence was confirmed by comparing the predicted restriction map to the map obtained from genomic DNA. Complete correspondence was found, establishing the validity of the sequence. Mapping of the transcriptional start point was accomplished using primer extension and was confirmed by nuclease protection. The sequence of the upstream region of the gp195 is shown in Figure 2 with the start site of transcription indicated by the flag. The transcription unit and the genomic DNA are continuous, indicating that trancription of this gene proceeds from a contiguous initiation site and proceeds through the gene. Features of eukaryotic promoters, such as TATAA and CAAT sequences are not observed. Instead, short repeated sequences are observed which are indicated by the boxes in the figure. The significance of those sequences is under evaluation (Lanzer and Ravetch, in preparation).

These initial studies answered several important questions regarding the transcription of plasmodial genes. Studies have been pursued in the ensuing years to define the transcriptional units of the KAHRP and GBP 130 genes, to determine the contribution of transcriptional and post-transcriptional events to the regulation of expression of these genes and to use the information derived from those studies to develop vector for DNA transformation. Progress in those studies are described in the reports for 1988-1989 and subsequently.

Fig. 1 Nuclear Run-on transcription assay for P. falciparum K^+ and K^- isolates.



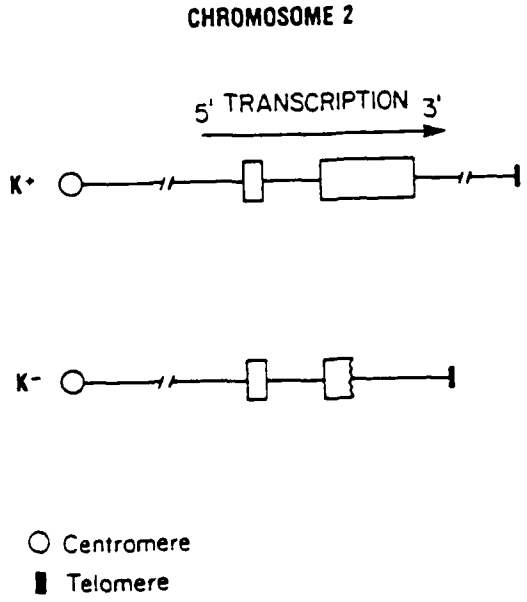
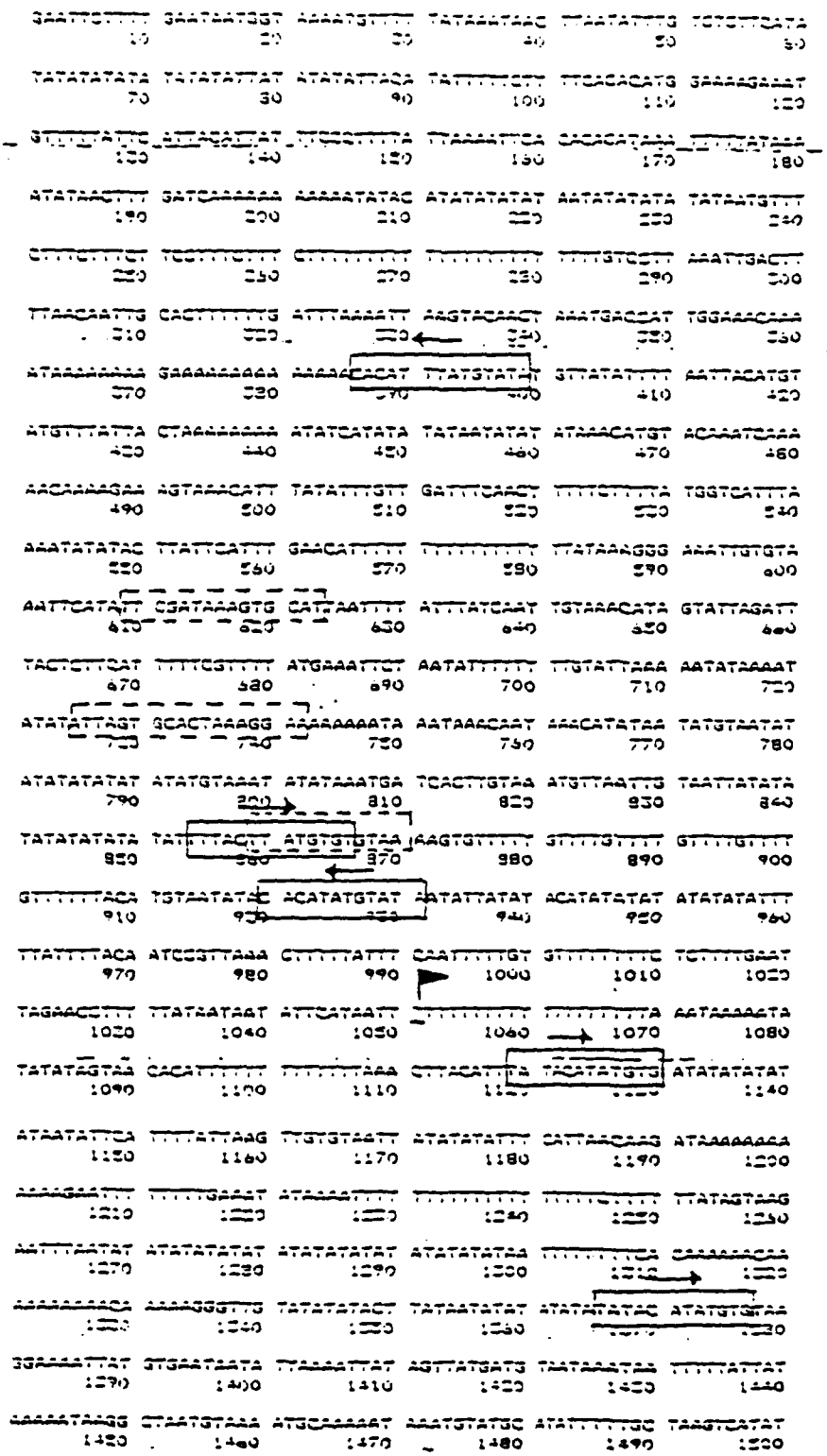
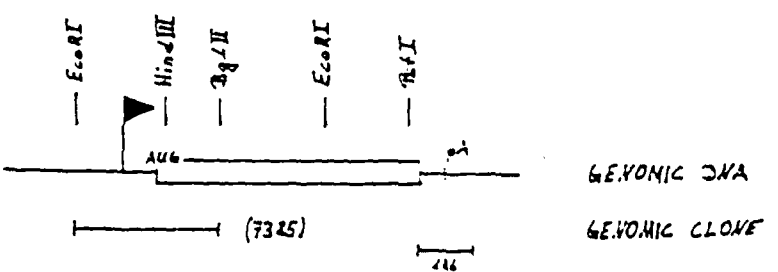


Fig. 2 Primary Sequence of the gp195 transcription initiation site and promoter region.



GENOMIC ORGANIZATION OF THE P195 LOCUS



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